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DEVELOPMENT OF AN IMMUNOASSAY FOR BACTERIAL ENDOTOXINS

ANNUAL/FINAL REPORT

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SEPTEMBER 1, 1986

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-82-C-2101

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: UNCLASSIFIED	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited.
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Scripps Clinic & Research Foundation; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Department of Immunology, IMM 12, 10666 North Torrey Pines Road, La Jolla, CA 92037	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: DAMD17-82-C-2101
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS: PROGRAM ELEMENT NO. 62772A; PROJECT NO. 3S1 62772A874; TASK NO. AA; WORK UNIT ACCESSION NO. 148

11. TITLE (Include Security Classification)

Development of an Immunoassay for Bacterial Endotoxins

12. PERSONAL AUTHOR(S)

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13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual/Final	FROM 4/15/82 TO 8/14/86	1986 September 1	15

16. SUPPLEMENTARY NOTATION

* Annual Report covers time period of April 1983 - August 1986.
Final Report covers time period of 15 April 1982 - 14 August 1986

17 COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	Keywords: ~~lipopolysaccharides~~ antibodies; lipopolysaccharide-binding-protein; lipopolysaccharide detection.
06	05		
06	01		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The goal of this study was to develop an assay for lipopolysaccharides (LPS) which would be as broadly sensitive to LPS from as diverse a group of gram negative bacteria as possible. Sixty different LPS reactive monoclonal antibodies were generated from Salmonella minnesota Re595 bacteria and germ-free mice. Cross reactive antibodies were screened for using a panel of structurally different LPS types. The data obtained resulted in a scoring system which allowed selection of several monoclonal antibodies as most likely to be useful in the LPS assay. During the antibody study a lipopolysaccharide binding protein (LBP) from rabbit acute phase serum was discovered and found to have broader LPS binding properties than the monoclonal antibodies. These results offer several strategies for development of a broadly sensitive LPS detection system. (SIW/KT)

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT ☐ DTIC USERS	Unclassified
22a. NAME OF RESPONSIBLE INDIVIDUAL: Mary Frances Bostian	22b. TELEPHONE (Include Area Code): 301-663-7325; 22c. OFFICE SYMBOL: SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

Introduction:

The major goal of this study was to develop an assay for lipopolysaccharides (LPS) which would be broadly sensitive to LPS from as diverse a group of gram negative bacteria as possible. To this end we sought to generate a population of murine monoclonal antibodies reactive with antigens common to all LPS. Two strategies for generating cross reactive antibodies were combined during mouse immunization: the use of heat killed Salmonella minnesota Re595 (Re595) as an antigen and the use of germ free mice for immunization. Re595 LPS is composed only of lipid A and an abbreviated core structure common to most, if not all, types of LPS. Germ free mice were chosen for immunization because they would not have been exposed to LPS prenatally and so should not have developed tolerance to any of the potential antigens of LPS.

Our strategy for antibody selection was to first screen hybridoma clones for reactivity with Re595 LPS. Those clones would then be rescreened for reactivity with E. coli J5 LPS, an LPS having more core structure than Re595-LPS, and with E. coli O111:B4 LPS, an LPS having a complete core and O-antigen. Antibodies reactive with this panel would then be screened for their LPS binding affinity and in other cross reactivity assays. Finally one or a few clones would be selected for larger scale antibody production and assay development.

While this approach to anti-LPS monoclonal antibodies was being pursued an event occurred which significantly altered the scope of our efforts; we observed that LPS binding protein (LBP) from acute phase rabbit serum would bind well to several types of LPS. Consequently, we explored whether an LPS assay based on the binding of LBP to LPS was

feasible. Early results with LBP and the antibodies suggest that our goal of a broadly sensitive LPS assay may be developed as easily with LBP as with anti-LPS antibodies.

Monoclonal antibody development:

Six germ free Balb/C mice were immunized intravenously over a three month period using acetone washed heat killed S. minn. Re595 bacteria until good serum antibody production was observed. Hybridomas were prepared by standard methods and the clones screened for anti-Re595 LPS secretion using an ELISA assay. Sixty clones were selected for further analysis of their specificity and antibody type. These results are summarized in Table 1. Antibody typing was performed with commercially available typing reagents. Of the sixty clones 9 were identified as IgM and 50 were identified as IgG2a, IgG2b, or IgG3. One culture, 07-10, appears to secrete both IgM and IgG3. Either this group of cells is not a monoclone, or the culture supernate was collected as the cells were undergoing a maturational change. Since we found that culture supernates did not provide enough antibody to test antibody LPS binding specificity ascites fluids were collected from a random sampling of the cell lines. These have now been studied in several ways. Immunoglobulin concentrations were determined by radial-immunodiffusion. The antibody binding specificity was initially tested using microtiter plates coated with LPS from either S. minn. Re595, (Re595-LPS), E. coli J5 (J5-LPS), or E. coli 0111:B4 (0111:B4-LPS) and determining the Ig concentration which gave half maximal binding. We refer to this as the B_{50} value. Binding specificity was also tested qualitatively in collaboration with Dr. T. Kirkland (Veteran's Administration, San Diego) using microtiter plates

coated with either LBS-bovine serum albumin conjugation (LPS-BSA) or with heat killed bacteria. Ascites fluids were diluted 100X, applied to the plates and the amount of immunoglobulin bound quantitated with an ELISA. Antibody binding in these assays was evaluated as either none, weak, medium or strong. The results to date are summarized in Table I.

Several conclusions may be drawn from these data. First, our method for detecting binding to LPS reveals that several of the antibodies bind quite well to all three types of LPS utilized. While no one clone is clearly "best" , clones 51-52, 29-17 and 22-19 give B_{50} concentrations lower overall than the other clones tested. One way of detecting this is to simply add the three B_{50} values for each clone to determine the overall B-50 score as given in the table. Dr. Kirkland's tests with LPS-BSA conjugates do not reveal either the cross reactivity our tests reveal or that Dr. Kirkland's tests with whole heat killed bacteria reveal. Taken together, these types of data provide a rational basis on which to eventually select several clones for further use in an LPS assay.

Lipopolysaccharide binding protein (LBP):

Work in our laboratory on the isolation of a new acute phase reactant has led to new and exciting possibilities for development of LPS assays. Briefly stated, we have observed that rabbit, mouse, and human acute phase sera all contain a component capable of binding to LPS. The rabbit protein has recently been purified, antibodies to it developed and studies of its properties begun. These results show that rabbit LBP is a novel acute phase reactant capable of direct binding to LPS in serum. While binding to Re595-LPS has been well documented we

have only recently explored the breadth of LBP's binding specificity. To do this samples of various types of LPS were spotted on genescreen membranes, the membranes soaked in solutions containing LBP and the binding of LBP to the LPS detec with a rat anti-LBP antibody. Examples of these results are shown in Figures 1-3. The detailed procedure is given in the Appendix. A positive result, i.e. LBP binding, is indicated by a dark spot on the genescreen membrane. Control experiments with heat inactivated LBP (75^{o}, 30 min; Figure 1) and with non-immune rat serum in place of rat anti-LBP antiserum establish that the positive results we see are due to LBP-LPS binding. The results shown in the Figure 2 establish that LBP is broadly reactive with a diverse spectrum of LPS types as well as with lipid A (Figure 2), and that LBP binds to LPS on the membrane either as a purified protein (Figure 1) or simply as a component of acute phase serum (Figure 2). The differential reactivity of LBP with the different LPS types may reflect the accessibility and content of lipid A. The association constant for binding must be quite high since LBP in whole acute phase serum appears to bind as well as isolated LBP. Finally, the spot which develops is sensitive to the amount of LPS (data not shown) and/or LBP (Figure 3) present.

These observations augur well for an LPS assay using LBP as the detection reagent. At this point it is difficult to predict the eventual sensitivity of the assay. One stratagem for improved sensitivity may be to briefly heat the LPS sample in dilute acid to free the lipid A from the rest of the structure.

It is important to note that our work on development of an immunoassay for LPS was stimulated by dissatisfaction with the limulus lysate assay for LPS. We believe it is very important that the newly realized potential of LBP as an LPS detection reagent be exploited. To our knowledge there is no other single reagent with as broad a reactivity for different types of LPS as LBP.

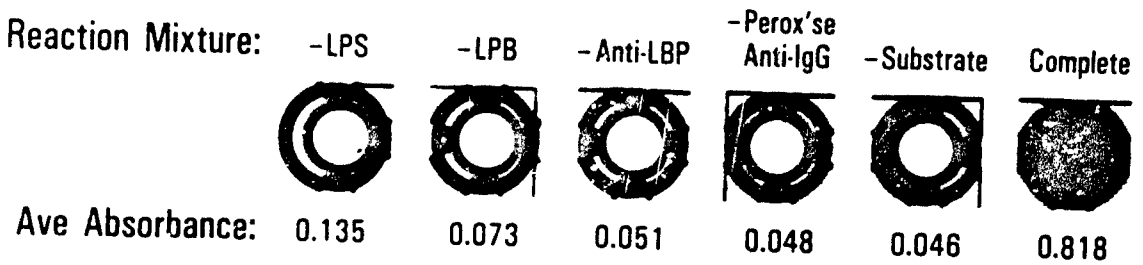
Reaction Mixture:
-LPS
-LPB
-Anti-LBP
-Perox'se Anti-IgG
-Substrate
Complete
Ave Absorbance:
0.135
0.073
0.051
0.048
0.046
0.818

Figure 1

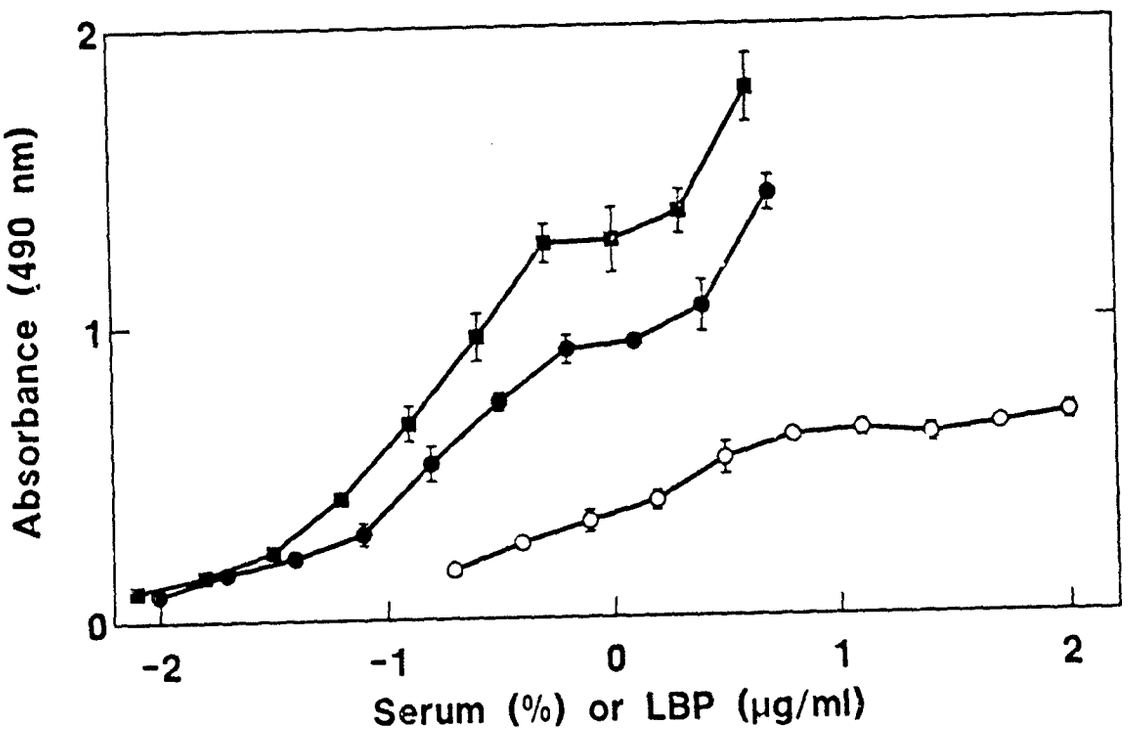

Figure 2

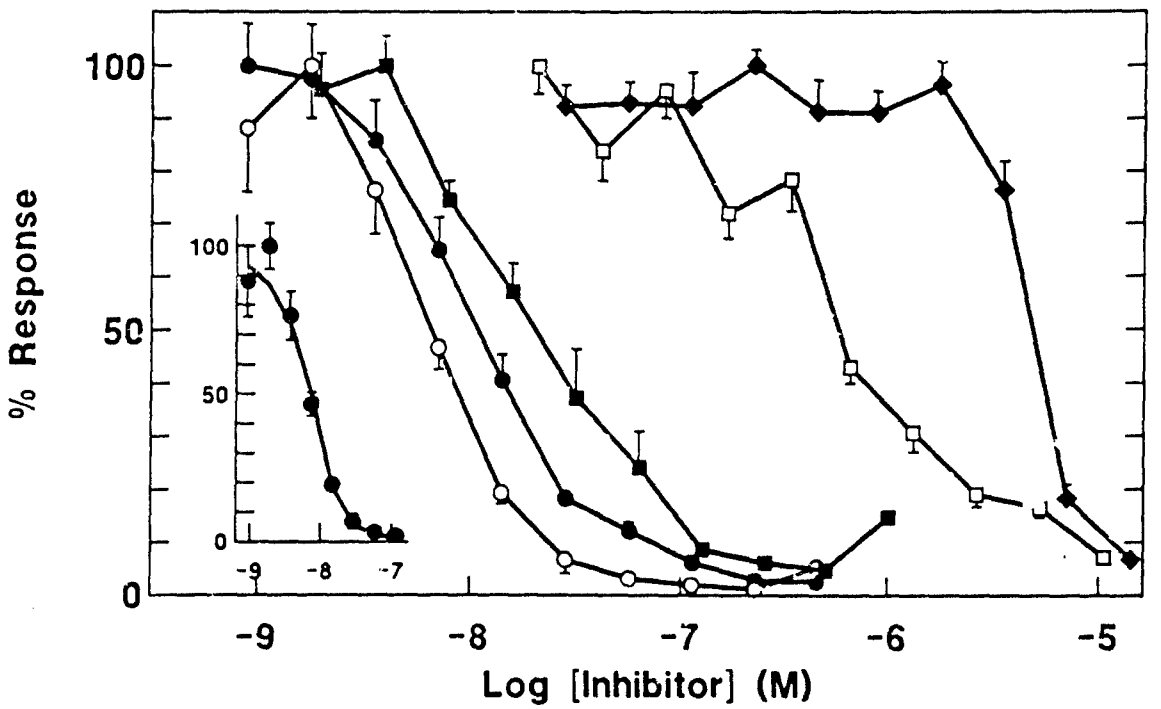

Figure 3a

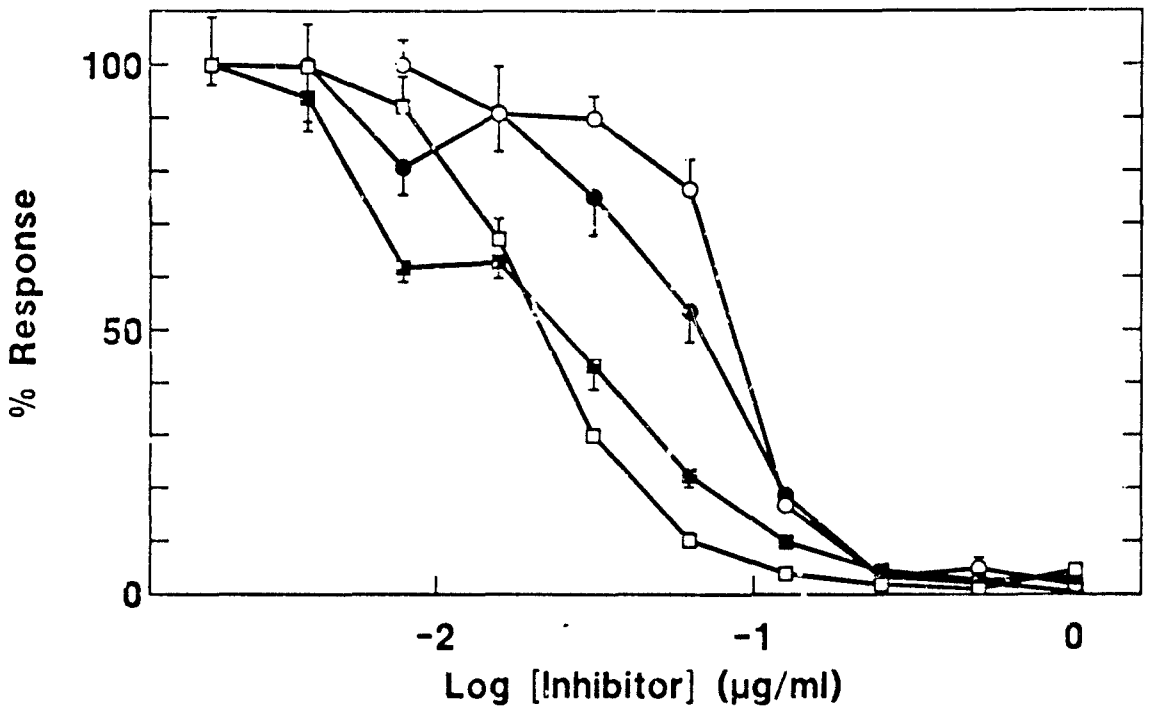

Figure 3b

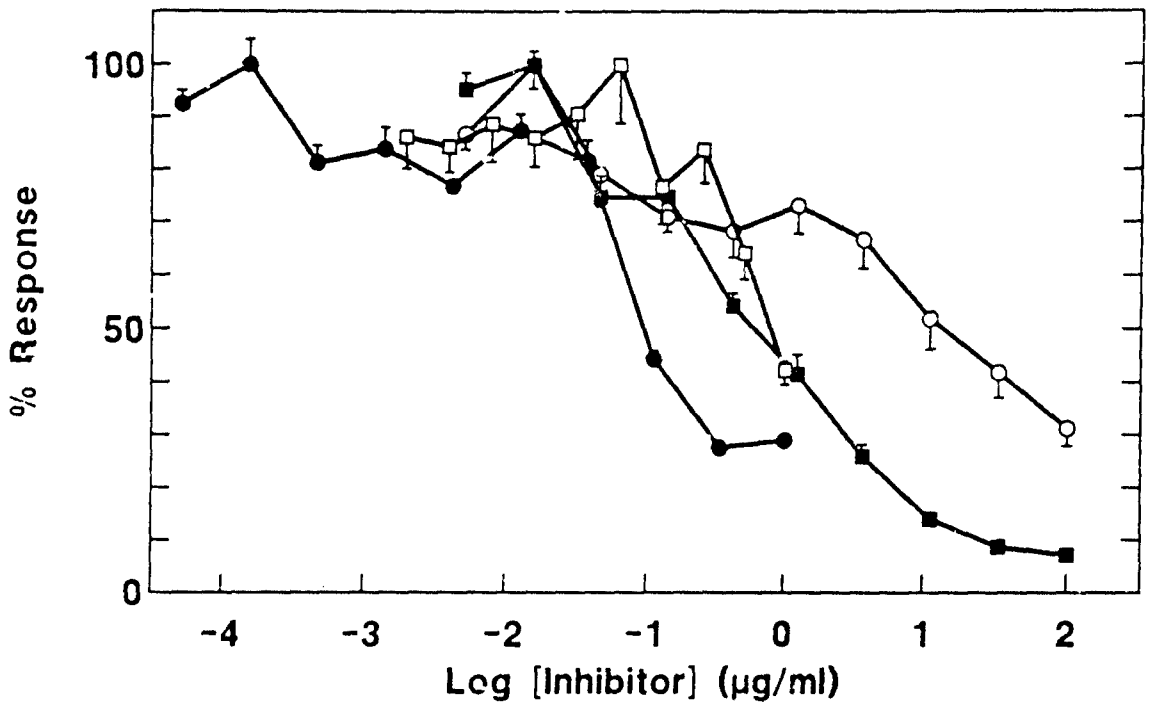

Figure 3c

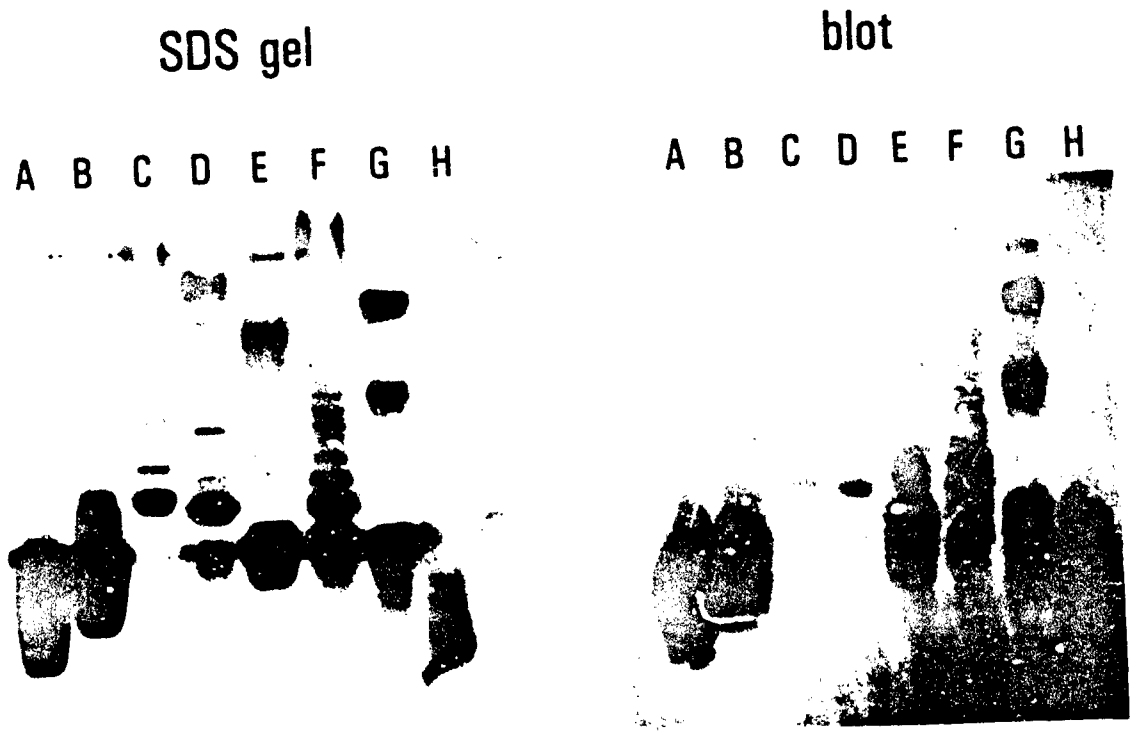

Figure 4

Table I

Current status of clones being studied:
19-Sep-86

1 = Yes
0 = No

B-50 values (ng/ml)

Reactivity with:

Scoring:

Abs	Stars
.00-.08	0
.08-0.4	1
0.4-0.8	2
>0.8	3

CLONE	IgM	IgG	IgG2a	IgG2b	IgG3	LPS: Re595	J-5	O111: B4	Score	BSA conjugate of J5 LipA	J5 LPS	Re595 LPS	E.coli-- ReLPS	RcLPS	killed bacteria ---S. minn.--- Ra	Rc	---E. coli--- Re595	J-5	O111: B4	04	Ser. keep.	Prot. 214	Pseud 388
07-04	0	1		1																			
07-10	1	1			1																		
07-17	0	1		1																			
07-25	0	1		1																			
07-34	0	1		1																			
10-01	0	1	1																				
10-21	0	1	1																				
10-44	0	1	1																				
10-50	0	1	1																				
11-01	0	1																					
11-06	0	1	1			>6000	-	-	>6000			‡‡‡	‡‡‡		‡		‡‡	‡	‡				
11-12	0	1	1																				
11-20	0	1	1																				
11-28	0	1	1			22	6.6	3.4	32			‡‡‡	‡‡‡		‡‡		‡‡‡	‡‡	‡	‡			‡
18-07	0	1		1								‡‡‡	‡‡‡		‡‡	‡	‡‡‡	‡‡	‡	‡			‡
19-02	0	1	1																				
19-25	0	1	1																				
19-47	0	1	1																				
19-49	0	1	1																				
22-19	0	1	1			12	11	1.4	24			‡‡‡	‡‡‡		‡		‡‡	‡	‡	‡			‡
23-01	0	1			1							‡			‡	‡	‡	‡	‡	‡			
23-12	0	1			1																		
23-15	0	1			1					‡	‡	‡‡	‡‡		‡		‡‡	‡	‡	‡			‡
23-16	0	1			1																		
23-25	0	1			1																		
23-3	0	1																					
27-01	0	1																					
29-04	0	1		1																			
29-07	0	1		1		24	2.7	1.3	28			‡‡‡	‡‡‡		‡		‡‡	‡	‡				
29-10	0	1																					
29-16	0	1																					
51-2	0	1	1																				
51-52	0	1	1			16	3	1.3	20			‡‡‡	‡‡‡		‡‡		‡‡	‡	‡	‡			
51-56	0	1	1			16	13	2	31			‡‡‡	‡‡‡		‡‡		‡‡	‡	‡	‡			
56-10	0	1	1																				
56-11	0	1	1																				
56-32	0	1	1																				
56-54	0	1	1																				
60-38	0	1	1									‡‡‡	‡‡‡		‡		‡‡‡	‡	‡				
67-18	0	1	1																				
67-20	0	1	1																				
69-23	0	1		1																			
69-27	0	1		1																			
69-60	0	1		1																			
70-24	0	1		1																			
70-28	0	1		1																			
73-18	0	1	1			110	5.6	2.9	118			‡‡‡	‡‡‡		‡‡		‡‡	‡	‡	‡	‡	‡	
73-30	0	1	1																				
73-64	0	1	1									‡‡‡	‡‡‡		‡		‡‡‡	‡					
76-26	0	1	1	1																			
84-10	0	1		1																			
10-14	1	0										‡‡‡	‡‡‡		‡		‡‡	‡	‡	‡			
16-03	1	0				25	6	2.7	34			‡‡‡	‡‡‡		‡		‡‡	‡	‡				
16-04	1	0				63	12	2.6	77			‡‡‡	‡‡‡	‡	‡		‡‡	‡	‡				
16-05	1	0																					
16-07	1	0				459	71	98	630			‡	‡		‡		‡		‡				
16-16	1	0																					
16-40	1	0										‡	‡		‡	‡	‡	‡	‡	‡			
16-44	1	0																					
16-53	1	0																					

Appendix

LPS Dot Blot Procedure using LBP:

1. Two ul LPS at 500 ug/ml, 50 ug/ml, or 5 ug/ml in 20 mM EDTA, 0.1 M sodium carbonate, pH 9.6, are spotted onto Genescreen membranes. The membrane is then dried at 80 degrees for 2.5 hrs.

2. Any remaining binding sites are blocked by soaking the membrane in 30 mg/ml BSA in 50 mM Tris, 0.2 M NaCl, pH 7.4 (TBS) for 15 mins at 37 degrees.

3. The membrane is then soaked in LBP solution (APRS or purified LBP at 10 ug/ml in TBS) overnight at room temperature. Excess LBP is washed away with 3-4 changes of TBS over a 30 min period.

4. The membrane is then soaked in rat anti-LBP, usually diluted 1000 fold, in TBS containing 1 mg/ml BSA for 3 hours at room temperature and rinsed as in step 3.

5 The membrane is then soaked in horseradish root peroxidase conjugated to goat anti-rat Ig for 1 hour at room temp and rinsed as in step 3.

6. The membrane is then stained with HRP color reagent (Bio-Rad) as directed by the manufacturer to reveal LBP binding.

7. Finally, the membranes are rinsed with water and air dried.